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**Daily Rhythmic Activity of the Sea-pen *Cavernularia obesa*
Valenciennes. XIX. Further Considerations on the Mechanism
of the Circadian Rhythmic Activity**

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With Text-figure 1

Abstract A general description of circadian rhythm of the sea-pen is given. The mechanism which supposedly maintains this circadian rhythm is explained. The periodic change of hydrogen ion concentration of body fluid which occurs with expansion and contraction of the animal is proposed as the mechanism which maintains the circadian rhythm of the sea-pen. This idea may be a kind of metabolic theory. Some difficulties for this theory are examined, and, despite these difficulties, the reality of the metabolic theory is again emphasized and the reasons for supporting this theory are explained. Some other considerations on the circadian rhythm of the sea-pen are also presented and the recent literature which seems to support the metabolic theory is reviewed.

Key words: circadian rhythm, metabolic theory, temperature compensation, sea-pen

Circadian rhythmic activity has been one of the biological phenomena of utmost concern and especially the mechanisms of inducing circadian rhythms have been an important object of discussion.

Broadly speaking, the author thinks there are two different views on the mechanisms underlying the circadian rhythms. Edmunds (1988) wrote in this connection: "Is the temporal ordering of developmental processes merely a causal chain of events wherein each successive event is caused by the completion of the preceding one, or are there also long-term mechanisms (clock, for example) which trigger the events at the proper time?" The former view of this notion has been proposed basing on a series of metabolic events observed, and the present author is holding this theory and has announced it with the experiments of the sea-pen, a lower invertebrate. Edmunds' latter clock theory is usually insisted by the investigators who are using higher animals such as insects, birds or mammals as research materials. Although many investigators have executed various experiments and expressed varied views concerning these two theories, the conclusive recognition which is effective through the whole animal world has not yet been reached.

In this paper, the author wishes to emphasize again the metabolic theory which seems to be effective for the maintenance of the circadian rhythmic activity of the sea-pen, by adding some new experimental data and referring to recent literature.

I. General sketch of the metabolic theory for maintaining the circadian rhythmic activity of the sea-pen

Since 1943 (Mori, 1943), the author has been studying the daily rhythmic activity of a sea-pen *Cavernularia obesa* Valenciennes and has published about 20 articles in a series. This animal shows a circadian rhythm which persists during even 100 days under constant darkness. The period of this free-running rhythm generally decreases slightly, and the periodicity of 18–21 hours becomes predominant. As for the mechanism which causes this circadian rhythm, the author proposed, preliminarily in 1944 (Mori, 1944) and definitely in 1945 (Mori, 1945a), that the rhythmic change of hydrogen ion concentration of body fluid related to the whole metabolism of the body is a very important concern. The author expressed this idea basing on the following facts.

(1) The sea-pen remains contracted in the bottom substrate during the daytime and starts to expand out above the substrate just after dusk. The animal expands larger and larger as time passes generally until midnight, and then suddenly begins to contract, disappearing before dawn from above the bottom. The time of contraction differs from colony to colony, ranging from about midnight to just before dawn, but the starting time of expansion is very much similar among colonies, so long as the environmental temperature is 15–30°C. Thus, the duration of expansion varies among colonies, from only 4–5 hours to 10–11 hours (Mori, 1945b).

With expansion and contraction of the colony the hydrogen ion concentration of body fluid changes rhythmically (Mori, 1944, 1960). It reaches the maximum (pH ca. 7.4) just before expansion behavior takes place and reaches the minimum (pH ca. 7.8) at the state of full expansion, just before the onset of contraction of the colony. The daily behavior of expansion and contraction in the sea-pen can be sketched as output of an automatic mechanism that maintains the circadian rhythm.

In the evening, the gradually accumulated hydrogen ions in the cells and tissues of the sea-pen through contracted state during daytime will stimulate the nervous system of the animal, and with removal of the inhibiting factor for expansion at the beginning of night, due to the lack of light, it begins to expand above the substrate. As the sea-pen expands, the seawater flows into the body, which dilutes body fluid and lowers its hydrogen ion concentration. With decrease of hydrogen ion concentration, the stimulation by the nervous system to keep expansion decreases; instead the stimulus to induce contraction increases. When the hydrogen ion concentration reaches the minimum, the sea-pen starts suddenly to contract.

(2) When a small amount (ca. 1–5 ml) of carbonated acidic seawater (pH 5.2) is injected through the top of the rachis of the sea-pen in the morning, that has drawn into the substrate a few hours ago, it begins instantly to expand in the same manner as at dusk (Mori, 1960). The change of hydrogen ion concentration of body fluid is presumed to stimulate the nervous system, leading to the expansion of the colony. It should be noted that this expansion occurs even in the light, which means that the stimulus by injecting acidic seawater is overwhelming the inhibiting stimulus by light. After several hours of expansion the injected colony begins to contract in the

same manner as the animal behaves naturally in the later half of the night (Mori, 1960).

The injection of seawater made acidic by mixing lactic acid or acetic acid also induces expansion of the sea-pen, but when the concentrations of these acids in seawater to be injected are beyond a certain level (more than 0.3% for lactic acid and more than 0.1% for acetic acid) the expanded animal becomes abnormal or rather morbid and the form of the rachis is usually distorted. The abnormality is greater when acetic acid is used for acidification of seawater than lactic acid is used, and the phenomena seem to suggest that there may be an influence of the CH_3COO^- or $\text{CH}_3\text{CHOHCOO}^-$ ions, because this abnormality is not encountered when carbonated seawater is injected.

(3) When the rachis is bisected from the top to its base (just above the stolon), as is shown in Fig. 1-a, both two halves of the rachis exhibit daily rhythmic expansion and contraction behavior as in a normal colony (Fig. 1-b, c). When 0.5 ml of seawater made acidic by mixing lactic acid (concentration 0.3%) is injected into one branch of the bisected rachis in contraction in the morning (Fig. 1-c), the branch expands as described in the preceding section (Fig. 1-d). The other branch shows a slight movement for a moment, but it returns soon to a normal contracted state and does not expand. The nervous stimulus caused by thrusting with an injection needle may be transmitted quickly to other branch, but it does not cause expansion of the non-injected branch (Mori, 1960). The results of the experiment indicates that the injected acidic seawater does not circulate through the stolon to the other, non-injected branch of the rachis.

(4) When the carbonated seawater or the seawater made acidic by mixing acetic acid is neutralized with a small quantity of liquid ammonia to make its pH value

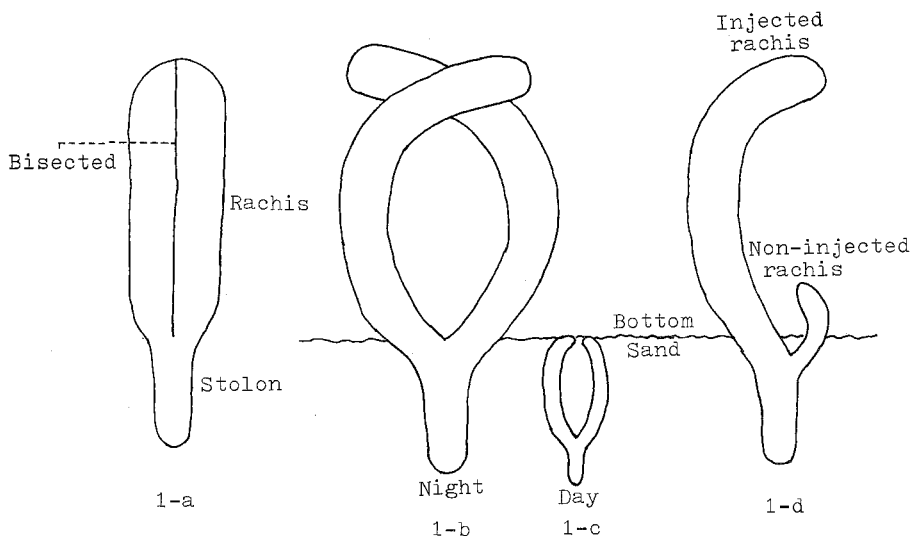


Fig. 1. Injection experiment of acidic seawater into one of the branches of the bisected rachis of a colony.

that of normal seawater (pH 8.3), its effect of injection disappears. This shows clearly that the change of hydrogen ion concentration is really effective for inducing expansion (Mori, 1960).

(5) The phase of the rhythm can be shifted by the injection of carbonated acidic seawater, which was clearly demonstrated by Imafuku (1973). When the carbonated seawater is injected into contracted sea-pens that have been cultured in constant dim light, they respond to the injection and expand as mentioned in the preceding sections. The subjective time of this expansion and contraction rhythm is fixed in the memory of the sea-pen, and following this memory the time phase of expansion and contraction rhythm is shifted to the experimental phase. This experiment seems also to offer a forceful base for the metabolic theory related to the fluctuation of the hydrogen ion concentration as the inducing factor for the circadian rhythm.

II. Difficulties to the author's metabolic theory

Several difficulties may be considered concerning the author's metabolic theory. They will be described below.

(1) Temperature independency. According to the author's experiments (Mori, 1945a, 1960), CO_2 content, ammonia content and hydrogen ion concentration of body fluid, glycogen content of body tissue and O_2 consumption of the animal, all these metabolic states show rhythmic changes in the course of a day. Usually the metabolic rate is dependent on the environmental temperature, and indeed Imafuku (1973) clarified that O_2 consumption rate of the sea-pen is within the usual range of Q_{10} (2.53).

Despite these facts, the circadian rhythm of the sea-pen is temperature independent so long as the environmental temperature is mild, that is, when maintained between 20–30°C. Basing on this fact of temperature independency, Imafuku (1973) doubts that the metabolic rhythm related to hydrogen ion concentration rhythm is the cause of circadian rhythm of the sea-pen.

(2) The author (Mori, 1945b), as well as Imafuku (1973), observed rhythmic activities of unusual manner; namely, some colonies showed two expansion states a night, while some others showed complete omission of an expansion state during a whole day. In some cases where an expansion state was omitted, the period of expansion of the following expansion lasted for 48 hours (i.e., twice a circadian period and not such as intermediate 30 or 36 hours). These phenomena appear to be difficult to be explained by a hypothesis that daily metabolic rhythm is causing the circadian rhythm.

(3) The author performed an experiment of injecting 1 ml of seawater made more alkaline than the normal seawater by mixing ammonia. The injection was executed in the morning just after the animal had contracted into the substrate, as was done in the case of injecting acidic seawater. In this experiment the pH values of seawater injected were 10.2 (0.01% ammonia), 10.8 (0.1%), 11.1 (0.2%) and 12.1 (0.5%). No effect was observed when the seawater containing 0.01% ammonia was injected,

similarly as injecting normal seawater. When 0.1% ammonia seawater was injected, the animal showed slight expansion but after 2 or 3 hours it contracted again. Under the injection of 0.2% ammonia seawater the colonies expanded gradually to about 10 cm above the substrate. The expansion was slow in comparison with the natural expansion process and the succeeding contraction did not occur for more than 10 hours and some colonies remained expanded even until the morning of next day (for more than 24 hours). The injection of 0.5% ammonia seawater made the animal totally abnormal and appeared to be fatal, and the colonies collapsed incidentally.

In summary, dilute solution of ammonia in seawater (less than 0.1%) induced the expansion of the colony of the sea-pen, but increased concentration of ammonia with higher pH values (more than 0.2%) elicited abnormal behavior of the colony and turned to be fatal. The increase of pH of body fluid appears to be related to the expansion of the colony of the sea-pen, notwithstanding that the pH increase is caused by unnatural way. However, the hydrogen ion concentration of body fluid of the sea-pen rarely exceeds 8.3, the results of injection of sea-water of much higher pH values may be argued as comparable to the natural processes the metabolic theory relies upon.

III. Re-emphasis of the metabolic theory

Despite several difficulties as mentioned in the preceding section, the author incline to emphasize again the metabolic theory related to rhythmic change of hydrogen ion concentration of body fluid as the causal mechanism for inducing the circadian rhythm of the sea-pen. The reasons for this re-emphasis are presented below.

(1) Problems about temperature independency. Temperature independency is said to be one of the most characteristic features arising with the circadian rhythm of organisms in general and the metabolic theory is, at first sight, holding some contradiction in this respect as described on Section II-1.

Many investigators have hitherto been discussing on the nature of temperature independency. Some considered that this problem was nearly solved by assuming the existence of some clocks independent of actual rhythmic metabolisms. For example, Page (1982, 1983a,b) and Tomioka & Chiba (1985), having examined cockroach and cricket respectively, concluded that the optic lobes were responsible for determining the free-running period, that is, acting as a pacemaking clock. Irrespective of the above report, Ikeda & Tomioka (1993) repoted later on the circadian locomotor rhythm in a cricket *Gryllus bimaculatus* and announced that both the pattern of daily rhythm and the free-running period were obviously altered by changing ambient temperature. They thought that these alterations were caused by masking of temperature compensation mechanism. Some other investigators, using mammals as research material, claimed that the hypothalamus of brain had a clock mechanism, and others expressed the idea for birds that the clock was located in the pineal gland (as for literatures refer to Edmunds, 1988).

However, in the lower animals such as coelenterates or in the unicellular

organisms, it is difficult to designate certain special organs which are acting as a clock, and some kinds of temperature compensation mechanisms should be postulated to explain the temperature independency. Palmar (1976) suggested that the component chemical reactions which maintained the circadian rhythms were either totally or partially temperature compensated. Edmunds (1976) also supposed that a series of compensatory reactions maintained temperature insensitivity. Loros and Feldmann (1986) found a remarkable fact that there was a temperature compensation mechanism in free-running rhythm in a kind of fungi *Neurospora crassa*, but they also found that there was a mutant strain in which the period of the rhythm was clearly affected by a change of temperature between 18–30°C, whose Q_{10} was approximately 2.

As mentioned before, the temperature independency of the expansion-contraction circadian rhythm of the sea-pen is observed only when environmental temperature is between 20–30°C, and below this temperature, especially below 15°C, it becomes temperature dependent (Mori, 1944). Under these low temperature conditions, about 20–40% of colonies remain contracted within the substrate every day, and in some case some colonies do not expand during consecutive 2 days, and once expanded there is a tendency to remain in this expanded state for as much as 24 hours. It seems nearly impossible for the sea-pen to maintain the circadian rhythm under these low temperature conditions. To put it more precisely, under these low temperature conditions, the circadian rhythm cannot be maintained under LL light condition, but under DD condition some colonies show circadian rhythm as long as for 8 days, after which the rhythm disappears.

The sea-pen does not show any circadian rhythm when it is reared at the temperature of about 10°C. When the rearing temperature is raised experimentally to about 22°C or 24°C, it recovers the original circadian rhythm. In natural habitat of the sea-pen in the coastal area of Shirahama on the southern Kii coast, these low temperature conditions (below 15°C) are encountered occasionally in winter. As for disappearance of the circadian rhythm under low temperature, Sweeney & Hastings (1960) reported that a marine dinoflagellate (*Gonyaulax polyedra*) lost the rhythm below 12°C, a temperature similar to that leading to the disappearance of the rhythm of the sea-pen.

Thus, when viewed over a wide range of temperature change, it can be said that the rhythmic behavior of the sea-pen is "temperature dependent," and within a limited range of mild temperature (20–30°C) some temperature compensation mechanisms may be operating to keep the circadian rhythm. The metabolic theory for keeping the circadian rhythm of the sea-pen may thus still be insisted effectively at present.

(2) Problems about abnormal modes of behavior. As the difficulties for the metabolic theory, several peculiar modes of the behavior were mentioned in Section II-2. The author is regrettable to say that there is no convincing way to explain these features on the base of the metabolic theory. However, these problems are very intricate and it is certain that no possible way of explanation will be found at present, even by the clock theory.

The following fact may have some relation to this phenomenon. In some cases, when a colony is contracted into the substrate, its several polyps are observed expanding above the surface of the substrate. In this state, the exchange of body fluid with surrounding seawater is taking place, no matter how much the amount of exchange is. When the seawater containing carmine is injected into the top of the rachis of completely contracted sea-pen, after a while the carmine-containing fluid is puffed out through the polyps at the top of the rachis. This shows that the outflow of body fluid occurs even when the sea-pen is contracted, presumably accompanied by intake of seawater of corresponding amount. If an interchange between body fluid and seawater is maintained to some extent, the omission of expansion of the colony can be brought about.

(3) Problems about the effect of injecting alkaline seawater. As stated in Section II-3, the injection of seawater made more alkaline than the normal seawater caused some kind of expansion of the contracted animal. Indeed, the change of hydrogen ion concentration of body fluid may cause expansion of the animal, but the manner of expansion seen when alkaline seawater is used is fundamentally different from that seen when used carbonated acidic seawater. The behavior of the animal in the latter case is quite normal, but that in the former case is fatally disturbed. It is not naturally expected that the hydrogen ion concentration in the body fluid of the sea-pen decreases than that of the normal seawater (pH 8.1–8.2) and so the injection of more alkaline seawater than the normal seawater is itself an unnatural experiment to demonstrate the natural response of the animal to the change of hydrogen ion concentration.

The author thinks there may be left several problems to be examined, but at present the results of injecting alkaline seawater may be neglected because they are done on unnatural supposition.

(4) Further bases in literature for supporting the metabolic theory. An intimate relation between the fluctuation of hydrogen ion concentration and the occurrence of circadian rhythm has been noticed and studied by several investigators. Chay (1981) reviewed the literature on this problem as follows. Gillies & Deamer (1979) said many biochemists and cell biologists had a great interest in the role of hydrogen ions in metabolic functions. Njus *et al.* (1974) and Sweeney (1974) reported that ion gradients play an important role in circadian rhythm and proposed a hypothesis that circadian rhythms are generated by a feedback mechanism comprised of ions and their associated membrane transport pathways. Their model qualitatively explains the temperature compensation phenomenon of circadian periods by the temperature adaptation of membrane lipids.

Chay (1981) himself presented a model and computation for oscillatory phenomena observed in some biological processes that utilize ion gradients across a membrane. His model has two main features: (i) active hydrogen ion transport pathways in the membrane and (ii) key enzymes having a pH-dependent activity profile and either translocating hydrogen ion from outside or producing hydrogen ion as a product. Sweeney (1987), showing a great interest in Chay's work, reported, in her

experiments principally on dinoflagellates: "ion transport across membranes and the synthesis of some proteins seem to be important, perhaps intimate parts of the circadian clock.... Although the circadian rhythm in the pulvinus of legumes depends on the coordinated changes in a number of cells, not just one, ion movement is certainly important in leaflet movement."

Concerning the above idea, Sweeney (1974) has previously proposed an interesting general automatic model that circadian rhythms are generated by a feedback loop, one component of which is active transport of one or several molecules concerned (x , which may be an ion such as K^+). When the molecules (x) are evenly distributed, the active transport into organelles is initiated. Transport of x into organelles continues until its concentration inside organelles becomes considerably higher than in the surrounding cytoplasm. In response to this high concentration of x , the configuration of the membrane proteins changes, so that active transport into organelles stops. Organelle membranes passively leak x at a slow rate all the time. When this is not opposed by active transport of x into organelles, the internal x slowly falls. When an even distribution of x is regained due to this leakage, active transport is reinitiated.

Furthermore, Palmer's (1976) summarization, presented after reviewing literatures together with his own experiments, is particularly noteworthy. He wrote: "Circadian oscillation is enzymatic activity and in numerous other biochemical parameters can and does occur in organs, tissues, isolated cells, and in unicellular organisms and at least some of these constituents most probably underlie (i.e., drive) the overt circadian rhythms. A basic assumption shared by many workers in the field is that this circadian clock has a cellular and biochemical origin." Similarly, Cloudsley-Thompson (1980) stated: "It is difficult to conceive of mechanisms, other than metabolic or hormonal ones, by which individual cells can synchronize with one another directly."

After examining the above and many other literatures it may be safely concluded that the hydrogen ion is intimately related to the maintenance of circadian rhythm of organisms and, although the author has no direct proof based on particular biochemical or enzymatic data, the hypothesis that the circadian rhythm of the sea-pen is maintained by, or at least closely related to, the rhythmic change of hydrogen ion concentration of body fluid, seems worth emphasizing still at present.

When viewed from another angle, the above hypothesis is tantamount to saying that the inducing cause is in the metabolic life rhythm of each cell and the condition of hydrogen ion concentration of whole body fluid is only an outcome of the metabolic life cycle of each cell.

IV. Several other considerations of the circadian rhythm of the sea-pen

(1) No connection between circadian rhythm and nutritional condition of the sea-pen. When the sea-pen is cultured for about 100 days in constant darkness under scarcity of food materials, the animal becomes very thin and small, yet the circadian rhythm persists. When a normal sea-pen expands fully at night, the length

of rachis (that is, the part of a colony standing out above the substrate) attains 30 or even 40 cm and its diameter becomes 4 or 5 cm; however, after 100 days' life in constant darkness under scarcity of food materials, the length and the diameter at the expanded state become about 10 cm and 1 cm, respectively. Despite this severe condition, the circadian rhythm of the sea-pen is insistently maintained.

(2) Hydrogen ion concentration of body fluid in winter. As described above, the times when the sea-pen starts to expand in winter relatively late in the evening, and the values of hydrogen ion concentration at that time are lower in comparison to those in summer. The hydrogen ion concentration of body fluid reaches pH of about 7.59–7.67 at the beginning of expansion in winter, in contrast to the summer value of 7.4. The author thinks that the low metabolic level in winter caused this low hydrogen ion concentration, and the expansion in the evening in winter was induced partly by the absence of light (which is a powerful inhibitory factor for the activity) irrespective of low hydrogen ion concentration in body fluid at that time. In this way, the daily rhythmic behavior is developed by a combination of the rhythmic change of hydrogen ion concentration and the rhythmic change of environmental light condition, in winter (at low environmental temperature) as well as in summer.

(3) Diversity of mechanisms to maintain circadian rhythms. The investigations on the circadian rhythms can be made from various directions using varied kinds of organisms. Naturally, the hypothesis on the mechanism maintaining each circadian rhythm may be diversified among investigators and from an organism used in certain experiments to other. For example, some investigators using mammals as materials say that they can find the source for controlling the circadian rhythm in the hypothalamus of brain, but others who are using insects say that the optical lobe is playing a key role for evoking the circadian rhythm. In these circumstances, Sweeney (1988) pointed out from her long experience that "it is dangerous to apply the experimental results from one organism to another." This can also be said to the difference in levels of organizations which the investigators are pursuing. Edmunds (1988) advocated: "it should become readily apparent that what constitutes a mechanism for one investigator is merely descriptive phenomenology for another working at a lower level of organization."

This indicates the difficulty of investigation in "biology" itself and at the same time the difficulty of identifying the real mechanism of circadian rhythm in general.

(4) After reviewing the relevant literature together with considering the author's own data, we must recognize, at least phenomenally, that some kind of temperature compensation mechanisms are acting widely in the living world. However, the author thinks that the fundamental or potential nature of rhythmic activity is temperature dependent in a broad sense, because there is no biochemical process being independent of temperature and the rhythmic activity is developed closely bound to internal biochemical processes. Whether the word "masking" of compensation is used or not, the temperature dependent phenomena of circadian rhythms are widely observed, and we should not overestimate the importance of temperature independency of circadian rhythm, notwithstanding the prevailing trend to place

its importance in the field of research concerned.

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